The Ttg2 ABC system in *Pseudomonas aeruginosa* functions as a general glycerophospholipid transporter between the two cell membranes

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Article

Keywords: Ttg2D, *Pseudomonas aeruginosa*, glycerophospholipid transporter, cell membranes

Posted Date: October 16th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-89298/v1>

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Version of Record: A version of this preprint was published at Communications Biology on April 9th, 2021. See the published version at <https://doi.org/10.1038/s42003-021-01968-8>.

Abstract

In *Pseudomonas aeruginosa*, Ttg2D is the soluble periplasmic phospholipid-binding component of an ABC transport system thought to be involved in maintaining the asymmetry of the outer membrane. The crystallographic structure of Ttg2D at 2.5 Å resolution reveals that this protein can accommodate four acyl chains. Analysis of the available structures of Ttg2D orthologs shows that they conform a new substrate-binding-protein structural cluster. Native and denaturing mass spectrometry experiments confirm that Ttg2D, produced both heterologously and homologously and isolated from the periplasm, can carry two diacyl glycerophospholipids as well as one cardiolipin. Binding is notably promiscuous, allowing the transport of various molecular species. *In vitro* binding assays coupled to native mass spectrometry show that binding of cardiolipin is spontaneous. Gene knockout experiments in *P. aeruginosa* multidrug-resistant strains reveal that the Ttg2 system is involved in low-level intrinsic resistance against certain antibiotics that use a lipid-mediated pathway to permeate through membranes.

Introduction

Pseudomonas aeruginosa are amongst the most important multidrug-resistant (MDR) human pathogens¹, showing inherent resistance to an important fraction of the available antibiotics². *P. aeruginosa* are responsible for chronic lung infections in individuals with chronic obstructive pulmonary disease or cystic fibrosis (CF)³ and account for over a tenth of all nosocomial infections⁴. A number of effective drugs and formulations can treat *P. aeruginosa* infections, even in CF patients⁵. These include frontline antibiotics such as piperazillin-tazobactam, ceftazidime, aztreonam, imipenem, meropenem, ciprofloxacin, levofloxacin, tobramycin, amikacin, and colistin⁶. Yet, resistance to most of these antimicrobials is being increasingly reported⁷. The basis for the inherently high resistance of these microorganisms is primarily their low outer-membrane (OM) permeability^{8, 9}, complemented by the production of antibiotic-inactivating enzymes (e.g. β -lactamases), the constitutive expression of efflux pumps^{10, 11} and the capacity to form biofilms^{1, 12}, among other mechanisms. The susceptibility of *P. aeruginosa* to antimicrobials can be additionally reduced by the acquisition of inheritable traits, including horizontal gene transfers and mutations that decrease uptake and promote efflux pump overexpression^{13, 14, 15}. Although a number of genes and mechanisms of resistance to antibiotics are already known in *P. aeruginosa*, the complex mechanisms controlling the basal, low-level resistance to these compounds are still poorly understood^{16, 17}.

The OM of *P. aeruginosa* is known to be central to its antibiotic-resistance phenotype. Its intrinsically low permeability is partly determined by inefficient OM porin proteins that provide innate resistance to several antimicrobial compounds, mainly of hydrophilic nature^{1, 8, 10}. In addition, studies with mutant strains have shown that the loss of specific efflux pump mechanisms, commonly overproduced in clinical isolates, is compensated by reducing the permeability of the OM⁹. Furthermore, polymyxin resistance in *P. aeruginosa* is associated with significant alteration of the membrane's glycerophospholipid composition¹⁸. Thus, mechanisms involved in OM organization, composition and integrity interfere with

the diffusion through the membrane of both hydrophilic and hydrophobic antimicrobial compounds. The asymmetry in the lipid organization of the OM is a main factor in the low permeability to lipophilic antibiotics and detergents¹⁹. Glycerophospholipid trafficking across bacterial membranes contributes to that asymmetry, but the systems involved in this transport and their directionality are just beginning to be understood.

In *Escherichia coli*, the Mla system (MlaA-MlaBCDEF) was initially proposed to have a phospholipid import function, preventing phospholipid accumulation in the outer leaflet of the OM and thus controlling membrane-phospholipid asymmetry²⁰. The core components of this ATP-binding-cassette (ABC) transport system in the inner membrane (IM) comprise the permease (MlaE), the ATPase (MlaF) and the substrate-binding protein MlaD that are highly conserved among Gram-negative bacteria²¹. The MlaA component, an integral OM protein that forms a channel adjacent to trimeric porins, would selectively remove phospholipids from the outer OM leaflet and transfer them to the soluble periplasmic substrate-binding protein MlaC^{22, 23}. MlaC would then transport the phospholipids across the periplasm and deliver them to MlaD for active internalization through the IM²⁴. Deletion of the genes of this system is known to destabilize the OM, and bacterial strains lacking any of the Mla components are more susceptible to membrane stress agents^{20, 25, 26, 27, 28, 29, 30, 31}. More recently, the retrograde transport hypothesis has been questioned and a new role for this system in anterograde phospholipid transport has been suggested. Thus, the Mla system in *E. coli* and its homolog in *Acinetobacter baumannii* appear to participate in the export of phospholipids to the OM^{30, 32}. In this context, MlaD would extract phospholipids from the IM and transfer them to MlaC in an ATP-independent manner.

The orthologous Mla system in *P. aeruginosa* is encoded by the PA4452-PA4456 operon (locus tags corresponding to PA01) and the isolated gene PA2800 (MlaA ortholog, also known as VacJ). Proteins encoded by this gene cluster are highly similar to those encoded by operon *ttg2* (toluene tolerance genes) in *Pseudomonas putida*^{33, 34}. Although it is unlikely that organic solvents themselves are substrates of this transporter, this system was initially linked to toluene tolerance in this species³⁵. Accordingly, components of the *P. aeruginosa* ABC transporter encoded by the PA4452-PA4456 have been named Ttg2A (MlaF), Ttg2B (MlaE), Ttg2C (MlaD), Ttg2D (MlaC) and Ttg2E (MlaB)³³. Recent studies of mutant strains with disrupted *ttg2* or *vacJ* genes support the contribution of this ABC transport system to the intrinsic resistance of *P. aeruginosa* to antimicrobials^{25, 29, 33, 36}. Nevertheless, one of these studies has challenged the role of this system in intermembrane phospholipid trafficking in *P. aeruginosa*³³.

Here, we have primarily focused on *P. aeruginosa*'s Ttg2D (Ttg2D_{pae}) and the nature of its cargo, which shall provide additional clues on the function of the Ttg2 system as a whole. Thus, we present structural and functional evidence of the role of this protein as a general phospholipid transporter, capable of carrying either diacyl or tetra-acyl glycerophospholipids. Our structural analysis further enriches the existing knowledge on the structural diversity of substrate-binding proteins (SBPs) and supports current discussions on the directionality of phospholipid transport by the Mla system. In addition, based on mutational studies of the *ttg2* operon, we have validated the contribution of the Ttg2 system to the

intrinsic basal resistance of *P. aeruginosa* to several antibiotic classes and other damaging compounds. Although the role of other components of this ABC transport system in multi-drug resistance has been already established for *P. aeruginosa*^{25,33}, this is the first study focusing on the soluble periplasmic SBP component, Ttg2D_{Pae}. Among the components of the Ttg2 system, this SPB could be the most promising candidate for an antimicrobial intervention based on the specific blocking of this trafficking pathway.

Results

Ttg2D_{Pae} contains a large hydrophobic cavity that binds four acyl tails

Sequence analysis indicates that Ttg2D_{Pae} (PA4453) is the soluble periplasmic SBP component of the ABC transporter encoded by the *ttg2* operon and a member of the Pfam family MlaC (PF05494). Interestingly, the available 3D structures for the MlaC family from *Ralstonia solanacearum* (PDB entry 2QGU), *P. putida* (PDB entries 4FCZ and 5UWB) and *E. coli* (PDB entry 5UWA) were all solved in complex with a ligand in their hydrophobic pocket, except for one structure from *E. coli* (PDB entry 6GKI) where the protein was delipidated. Electron densities for the ligand were in all these structures compatible with phospholipid moieties, supporting their predicted role as phospholipid transporters. A sequence alignment shows that some of the residues thought to be involved in phospholipid binding in the *R. solanacearum* Ttg2D structure are conserved in the *P. aeruginosa* ortholog (Fig. S1). Remarkably, the electron densities for *P. putida* Ttg2D revealed the presence of two diacyl lipids in its pocket²⁷.

To investigate ligand binding at the molecular level, we determined by molecular replacement the crystallographic structure of the Ttg2D_{Pae} mature protein (without the signal peptide, aa 23–215) produced in *E. coli* at 2.53 Å resolution (PDB entry code 6HSY) (Fig. 1A). The structure was refined to a final R_{work} and R_{free} of 20.9 and 24.9%, respectively, and good validation scores (Supplementary Table S1). All residues but the last three C-terminal ones (plus the C-terminal expression tag) could be modeled. Ttg2D_{Pae} adopts a mixed $\alpha + \beta$ fold with a highly twisted anti-parallel β -sheet formed by five strands and surrounded by eight α -helices. It exhibits a “decanter” shaped structure never described before for any other protein family (Fig. 1A). The structure presents a highly hydrophobic cavity between the β -sheet and the helices that spans the whole protein and has a volume of 2979 Å³ and a depth of ~25 Å (Fig. 1B). After the first refinement stage (AutoBuild), without any ligand added, clear density was visible inside the cavity that could correspond to four acyl chains (Fig. S2). We therefore modeled inside the cavity two PG(16:0/cy17:0) (Fig. 1A), as mass spectrometry (MS) experiments suggested that this lipid was one of the most abundant among those found to bind Ttg2D_{Pae} when expressed in *E. coli* (see below). Real-space correlation coefficients of 0.9 for the lipids indicate a good fit to the $2mF_o - DF_c$ electron density. The four acyl tails are deeply inserted into the hydrophobic cavity, while the polar head groups are exposed to the solvent and make only few contacts with the protein (Fig. 1, A and C). This lack of specific recognition of the head group could explain why Ttg2D_{Pae} is able to bind different types of

phospholipids. The presence of two diacyl lipids suggests that the protein could also be able to bind one tetra-acyl lipid, such as diphosphatidylglycerol (cardiolipin).

To investigate the mechanism of entry and release of the two lipids in the cavity of Ttg2D_{Pae}, we performed a normal mode analysis (NMA). NMA may be used to model the internal collective motions of a protein, relevant to ligand binding and function in general, typically described by a few low-frequency modes³⁷. Figure 1D shows the collective motions along mode 7, the first non-trivial mode (modes 1 to 6 account for translational and rotational motions of the protein as a whole). Rather than "en bloc" relative motions of sub-domains, all secondary structures of the protein appear to move in a concerted manner, helix α_4 and the core of the β -sheet being more rigid. This breathing-like motion increases in a concerted manner the volume of the cavity and its mouth area, and may allow the lipids to enter into or exit from the cavity. Inspection of the next 10 lowest-frequency normal modes shows similar concerted motions. The recent MalC structure with no lipid bound (PDB entry 6GKI)³² shows similar collective motions along all modes, with similar amplitudes, indicating that the cavity could open and close in the absence of lipid. The normal modes can be also used to compute atomic mean-square displacements, which can be in turn related to *B*-factors³⁸. The NMA-derived and the observed (crystallographic) *B*-factors are closely correlated except in regions 75–95 and 180–200, which are involved in crystal contacts, and region 105–120, where the electron density is weaker (Fig. S3). This suggests that the normal modes provide a realistic description of the protein's flexibility.

The Ttg2D/MlaC fold: a new two-domain architecture and SBP structural cluster

All MlaC homologs of known structure have a highly superposable "decanter" shaped configuration (Fig. 1A), previously described as an "extended" NTF2 fold²⁷ but never assigned a distinct structural classification. Thus, while the *P. putida* structure (PDB entry 4FCZ) appears in the CATH database as a single domain protein and unique structure of superfamily 3.10.450.710, belonging to fold topology 3.10.450 (Nuclear Transport Factor 2; Chain: A), the *R. solanacearum* structure (2QGU) is described as a two-domain structure with domains belonging to CATH-superfamilies 3.10.450.50 (NTF2-like) and 1.10.10.640. The latter superfamily belongs to the all-alpha fold topology 1.10.10 (Arc Repressor Mutant, subunit A) and has 2QGU as unique structure. To clarify the structural classification of Ttg2D/MlaC proteins we run a DALI search of the putative second domain (D2 in Fig. S1) of Ttg2D_{Pae} against the whole PDB. The results showed very good superposition with the small alpha domain of several AAA+ proteins, the best match being with 6UKS chain C³⁹ (Fig. S4). AAA (ATPases Associated with diverse cellular Activities) domains are formed by a large N-terminal domain adopting a Rossmann fold and a small C-terminal domain forming an alpha-helical bundle. They tend to adopt homo-hexameric ring complexes and hydrolyze ATP to perform activities that involve protein remodeling. The helical domain plays an important role in coupling the conformational changes resulting from ATP hydrolysis to the neighbor monomer within the AAA-ring and to the underlying protease ring⁴⁰ and has a specific classification (other than AAA) both in the PFAM (PF17862) and CATH databases (1.10.8.60). Interestingly, the Ttg2C ortholog MlaD, which in *E. coli* interacts with MlaC³², forms also a homo-

hexameric ring. We therefore conclude that currently known Ttg2D/MlaC structures combine two structural domains in an architecture not seen yet in other proteins, a NTF2-like domain and an AAA helical-bundle domain. In Ttg2D_{Pae}, the first domain is formed by two non-contiguous sequence segments: D1S1 (PDB residues 23–68), with three alpha-helices, and D1S2 (113–169), with five beta-strands. The second domain, with five helices, is also split in two non-contiguous sequence segments: D2S1 (69–112) and D2S2 (170–212) (Fig. 1A and S1).

Classical SBPs linked to ABC transporters are structurally similar and composed of two globular domains formed by discontinuous segments⁴¹, adopting either a Rossmann fold (CATH: 3.40.50) or a very similar CATH: 3.40.190. The 501 SBP structures available in the PDB have been classified in 7 clusters and several subclusters⁴². We superposed the Ttg2D_{Pae} structure to a representative of each of these subclusters. RMSD values, number of aligned residues and structural superpositions are shown in Fig. S5. The longest match aligns 47 residues with an RMSD of 3.97 Å (2PRS chain A, a 284-residue member of cluster A-I), while the best RMSD is 1.41 Å with 23 residues aligned (3MQ4 chain A, a 481-residue member of cluster B-V). These results clearly confirm that Ttg2D/MlaC family proteins do not belong to any previously known SBP structural cluster.

Evolution of sequence and structural diversity of the MlaC family

A structural alignment of MlaC family proteins with known 3D structures (Fig. 2A) reveals that, despite sequence identities ranging from 63% for the *P. putida* protein to as low as 17% for the *E. coli* one, the RMSDs of the structural alignments are very low, ranging from 1.6 to 3.1 Å (188 to 185 Cα), respectively (Table S2). Clearly, secondary structure elements are highly or strictly conserved among all four proteins, despite substantial amino-acid variation (Fig. 2A and S1). However, the four proteins split into two groups: *P. aeruginosa* and *P. putida* Ttg2D have a hydrophobic cavity of 2979 – 2337 Å³ and can bind two diacyl lipids, while the *R. solanacearum* and *E. coli* proteins have a half-size cavity of 1444 – 1332 Å³ and bind only one diacyl lipid (Table S2). Surprisingly, although the different number of ligands had been already noticed when the structure of Ttg2D from *P. putida* was solved, cavity differences were never analyzed. Figure 1B illustrates the cavity difference between *P. aeruginosa* and *R. solanacearum* Ttg2D. The volume differences correlate with the different number of residues forming the cavities, from 55 down to 31 (Table S2). However, these residues, which are spread along the whole protein sequence (Fig. S1), are largely conserved in terms of position and, in most cases, in terms of identity or similarity, with a few substitutions such as V147/L, or V163/I or M directly affecting the volume. Some side-chain reorientations, like Y105, and small secondary structure displacements, like strands β3 and β4 or helix α6 shifted by ~ 2 Å (Fig. 2A and S6), also modulate the volume. Taken together these changes are, nevertheless, not sufficient to explain how the cavity volume can double. Helix α8 seems to be the main responsible for the difference between a two and a one diacyl-phospholipid cavity, not only because the helix is longer in the first case (Fig. S1), but also because it adopts a different conformation. Indeed, for the second group (*R. solanacearum* and *E. coli*, one diacyl lipid), this helix has a straight conformation, covers the α6 helix (Fig. 2A and S6) and does not participate in the cavity (Fig. 1B and S1), while in the

first group (*P. aeruginosa* and *P. putida*, two diacyl lipids), the $\alpha 8$ helix is bent towards and over the $\alpha 7$ helix and greatly enlarges the cavity (with additional residues from $\beta 4$ and $\beta 5$ strands). This bend occurs at residue G195 with an angle of 40° and 64° in Ttg2D proteins from *P. aeruginosa* and *P. putida*, respectively (Fig. 2A). The helix of the first protein has an additional bend of 43° at K202. Glycine has a poor helix-forming propensity⁴³ and tends to disrupt helices because of its high conformational flexibility. On the other hand, the Phe197 and Gln196 residues occupying the Gly195 position in the *R. solanacearum* and *E. coli* proteins, respectively (Fig. S1), have better helix-forming propensities and maintain the $\alpha 8$ helix straight. In addition, W196, exclusive of the pseudomonal structures, may also contribute to the influence of the $\alpha 8$ helix on the cavity's volume, since its bulky hydrophobic side chain, deeply inserted into a hydrophobic pocket on the concave side of the curvature, could stabilize the helix $\alpha 8$ bend (Fig. S6).

Components of the Mla system are broadly conserved in Gram-negative bacteria, except for the periplasmic MlaC that notoriously shows high inter-species sequence diversity (Fig. 2B). Interestingly, an alignment of 151 representative amino-acid sequences belonging to the MlaC family and identified across different Gram-negative species revealed that W196 is conserved not only in *Pseudomonas* species but also in a group of related sequences in other non-phylogenetically related gamma-proteobacteria (Fig. 2C). In this group of proteins that would hypothetically bind two diacyl phospholipids, other positions with distinct residues relative to other MlaC family members stand out, especially in two regions located between the central part and the C-terminal end of the protein (Fig. S6). Side-chain orientation and hydrophobicity of some residues in these regions could be also contributing to a tighter binding of the two diacyl phospholipids inside the ligand cavity. The presence of common protein sequence signatures in species that are not closely related indicates that horizontal gene transfer, mediated by recombination events between flanking conserved genes, could have contributed to MlaC family diversity.

Ttg2D_{Pae} binds two diacyl glycerophospholipids and cardiolipin, representing a novel phospholipid trafficking mechanism among Gram-negative bacteria

Native MS was used to determine the lipids that bind specifically to recombinant Ttg2D_{Pae} produced in the cytoplasm of *E. coli* and the stoichiometry of the interaction in a cellular environment (Fig. 3). The native mass spectrum of Ttg2D_{Pae} shows two major charge states (m/z 2701, $z = 9$ and m/z 2430, $z = 10$) corresponding to a deconvoluted average mass of 24296 Da that matches the MW of the recombinant protein Ttg2D_{Pae} produced in *E. coli* without the first methionine residue plus bound ligands (Fig. 3A and S7). After isolation of the wide peak ion at m/z 2700 ($z = 9$) and gas-phase fragmentation with a transfer collision energy (CE) of 50 V, we detected the unbound protein (m/z 2536, $z = 9$) and a family of released phospholipids in the low mass range (Fig. 3B-C). Additional native MS experiments on isolated ion m/z 2700 ($z = 9$) using increasing transfer CE confirmed that at least a fraction of the bound Ttg2D_{Pae} population hosts two phospholipids, since lipid dissociation starts at a transfer CE of 35 V and goes through the single-bound species (Fig. S8). The results also show that a second fraction of the

Ttg2D population hosts a single molecule, that remains bound up to a transfer CE of 50 V, which could correspond to cardiolipin as further MS experiments indicated. A tighter binding of cardiolipins could explain why these molecules were not detected in the gas-phase fragmentation experiments (Fig. 3B-C). We also note that at the transfer CE required for cardiolipin release these molecules are easily fragmented (see below).

The mass of $[M + H]^+$ ions at m/z 664.5, 704.5, 718.5 and 730.5 released from Ttg2D_{Pae} confirmed the identity of some ligands as phosphatidylethanolamines (PE) with different hydrocarbon chains (PE C30:0, PE C33:1, PE C34:1 and PE C35:2 respectively) (Fig. 3C). Subsequent direct MS analysis under denaturing conditions in both positive and negative ion modes, identified additional species of PE and of other phospholipid classes that are also components of the bacterial membrane, such as phosphatidylglycerol (PG), phosphatidylcholine (PC) and phosphatidylserine (PS) (Fig. 3D and S9). With these methods, PG C33:1, PG C34:1, PE C33:1 and PE C35:1 came out as most abundant (annotation of lipid species based on the most abundant fatty acids in *E. coli* is given in Table S3). The observed distribution of phospholipids bound to recombinant Ttg2D_{Pae} relates to their relative abundances in *E. coli* BL21 (the recombinant protein-expression host) as determined by lipidome analyses (Fig. S10), and it correlates well with the reported phospholipid composition of *E. coli* under comparable conditions^{44, 45}.

LC-MS analysis under denaturing conditions in positive ion mode of the complexes produced in *E. coli* shows lipid species of m/z 690–800 and m/z 1400–1500 that could correspond to phospholipids and cardiolipins, respectively (Fig. S9D). In addition, direct MS analysis in negative ion mode indicates the presence of glycerophospholipids and shows peaks at m/z 1050–1160 that could correspond to *E. coli* cardiolipin species having lost one fatty acid (Fig. 3D-E). During the fragmentation processes of phospholipids in negative ion mode, upon low energy collisional activation, ions corresponding to the loss of fatty acids are the most abundant⁴⁶. For example, when the most abundant cardiolipin species in *E. coli*, i.e. CL C68:2 with MW 1405.0 Da (Fig. S10), loses one of the fatty acids at position *sn*-2, it shows a prominent fragment ion at m/z 1147 or 1121 depending on the fatty acid species in that position (Fig. 3D-E).

In light of these results, we performed additional native MS experiments to investigate whether delipidated Ttg2D_{Pae} can bind cardiolipin *in vitro*. In Fig. 4, representative native mass spectra of a reaction mixture containing 26.25 μ M delipidated protein and 245 μ M cardiolipin CL(18:0)₄ (1:9 molar ratio) in a buffered aqueous solution, are shown. The protein–cardiolipin complex and the unbound protein were detected around the 10+ and 9+ charge states (Figs. 4 and S11). Notably, the dissociation energy needed to release cardiolipin was higher than that required to release diacyl glycerophospholipids in the native complex (transfer CE of 60 V vs 35 V, see Figs. S11A and S8, respectively). This correlates with the previous observation that a "P + 2PL" population in the native spectra in Fig. S8 loss its cargo only at 60 V. At the transfer CE required for cardiolipin release this molecule is in fact easily fragmented (Fig. S11B).

Ttg2D_{Pae} binds two diacyl glycerophospholipids or cardiolipin in the periplasm of *P. aeruginosa*

Given that the cytoplasm of *E. coli* is clearly not the natural environment of Ttg2D_{Pae}, we decided to produce and purify this protein directly from the periplasmic space of *P. aeruginosa*. For this purpose, protein Ttg2D_{Pae} tagged with six C-terminal histidine residues and containing its own N-terminal secretion signal was expressed in the genetic background of a *P. aeruginosa* PAO1 $\Delta ttg2D$ mutant using a new derivative of a broad-host-range cloning vector (Fig. 5A). Restoration of the colistin susceptibility phenotype after transformation (MIC of 0.25 $\mu\text{g/ml}$ vs. 0.0625 $\mu\text{g/ml}$ in the non-transformed mutant) was used as evidence that the protein being expressed in the mutant strain was functional. Under these conditions, the mature Ttg2D_{Pae} could be purified from the periplasm in sufficient amount and purity (Fig. 5B inset) to determine its phospholipid cargo by MS.

The native mass spectrum in Fig. 5B shows a charge-state distribution that corresponds to the ligand-bound mature Ttg2D_{Pae} complexes (MW of intact complex: 23621 Da; MW of His-tagged protein without the signal peptide: 22140 Da). The wide peak widths, besides poor desolvation in aqueous buffer in the native MS instrumental conditions, suggests also the coexistence of multiple species of similar mass, supporting the presence of different classes of phospholipids bound to the protein. Gas phase dissociation mass spectra at varying transfer CE of isolated ions around $m/z = 2625$ ($z = 9$) indicated the presence of two populations at peaks $m/z = 2626$ ($z = 9$) and $m/z = 2619$ ($z = 9$), which are in agreement with the binding of two phospholipids and one cardiolipin, respectively (Fig. 5C). Further MS experiments under denaturing conditions in negative ion mode allowed the identification of two main phospholipid classes (Fig. S12 and Table S4). The obtained distribution is in agreement with relative abundances in *P. aeruginosa* PAO1 as determined by a lipidome analysis (Fig. S10), and it correlates well with the reported phospholipid composition in this species⁴⁷.

The Ttg2 system provides *P. aeruginosa* with a mechanism of resistance to membrane-damaging agents

As expected, the *P. aeruginosa* $\Delta ttg2D$ mutant exhibited a debilitated outer membrane leading to increased susceptibility to several membrane damaging agents (Fig. 6), as demonstrated by the 1-N-phenylnaphthylamine (NPN) assay. Indeed, an enhancement in NPN uptake was observed in the mutant in the presence of the permeabilizer agents EDTA and colistin (Fig. 6, A and B). In line with this, the $\Delta ttg2D$ mutant is significantly more susceptible to the action of polymyxins (lipid-mediated uptake), but also of antibiotics that use both the lipid- and porin-mediated pathways to penetrate the cell, including fluoroquinolones, tetracyclines and chloramphenicol (Fig. 6C). With regard to polymyxin antibiotics, the $ttg2D$ transposon insertion mutant was eight-fold more susceptible to colistin than the PAO1 wild-type, a colistin-susceptible reference strain (Table S5). In general, the mutation did not significantly affect the resistance phenotype displayed by the PAO1 strain to the beta-lactam antibiotics or aminoglycosides tested. The susceptibility phenotypes due to deletion of $ttg2D$ could be fully or partially reverted by complementation with the cloned $ttg2D$ gene or the full operon $ttg2$ in the replicative broad-range vector pBBR1MCS-5 (Fig. 6 and Table S5), confirming the link between the gene and the phenotypes. We have also confirmed that insertional mutations in each of the other components of the $ttg2$ operon ($ttg2A$, $ttg2B$, $ttg2C$) and $vacJ$ ($m1aA$ ortholog) lead to an increased susceptibility to antibiotics in the same way

as for the $\Delta ttg2D$ mutant (Table S5). The $\Delta ttg2D$ mutant is also significantly susceptible to the toxic effect of the organic solvent xylene (Fig. 6D) and it is four-fold more susceptible to the chelating agent EDTA (MIC = 0.5 mM) than the parental wild-type PAO1. However, no difference was observed between the mutant and wild-type cells in their susceptibility to SDS, obtaining for both strains a MIC value of 0.8%. Finally, disruption of the *ttg2D* gene resulted in an approximately two-fold reduction in biofilm formation and greatly increased the activity of EDTA against *P. aeruginosa* biofilms at a subinhibitory concentration of 0.05 mM (Fig. 6E).

The Ttg2 system is associated to *P. aeruginosa*'s intrinsic resistance to low antibiotic concentrations

The susceptibility of Ttg2-defective mutants to antibiotics was further studied in strains with different genetic backgrounds. To this end, the full *ttg2* operon was mutated in the clinical MDR *P. aeruginosa* strains C17, PAER-10821 and LESB58, which had shown different patterns of resistance to several antibiotic classes, specifically, polymyxins, fluoroquinolones and tetracyclines (Table 1). In particular, PAER-10821 and LESB58 are *P. aeruginosa* strains with low-level resistance to colistin. The generation of mutants with disrupted gene functions in MDR bacteria is troublesome because the antibiotics commonly used in the laboratory are no longer useful for selection of gene knockouts. In addition, the loci mutated in this case is involved in a general mechanism of resistance to antimicrobial agents and mutant strains are therefore expected to be generally susceptible and thus potentially lost during the selection steps. For this reason we have adapted a mutagenesis system based on the homing endonuclease I-SceI^{48, 49} to construct targeted, non-polar, unmarked gene deletions in MDR *P. aeruginosa* strains (see material and methods, text S1 and Fig. S13 for details). With this modified mutagenesis strategy we have obtained and validated unmarked deletion mutants of the selected MDR strains lacking the full *ttg2* operon (Fig. S13). Complemented strains were also obtained by transformation of mutant strains with a replicative plasmid containing the full *ttg2* operon and its expression in the complemented clones was confirmed by RT-PCR (Fig. S13). All these strains were tested for their susceptibilities to different classes of antibiotics (Table 1).

Table 1
Antibiotic susceptibility profile of *P. aeruginosa* MDR strains lacking the full *ttg2* operon.

Antibiotic	MIC [†] in µg/ml					
	LESB58		C17		PAER-10821	
	WT	$\Delta ttg2$	WT	$\Delta ttg2$	WT	$\Delta ttg2$
Polypeptides						
Colistin	4	0.125*	2	0.125*	32	32
Fluoroquinolones						
Ciprofloxacin	2	1	256	64*	256	128
Levofloxacin	8	2*	256	256	256	128
Ofloxacin	16	4*	> 32	> 32	> 32	> 32
Norfloxacin	8	4	> 256	> 256	> 256	256
Tetracyclines						
Tetracycline	16	8	32	16	32	8*
Minocycline	32	8*	16	8	32	8*
Tigecycline	16	8	64	8*	32	8*
Chloramphenicol						
Chloramphenicol	32	32	128	64	128	64
Sulfonamides						
Trimethoprim-sulphamethoxazole	16	8	> 64	> 64	> 64	64
Aminoglycosides						
Tobramycin	8	2*	64	128	128	> 128
Amikacin	64	64	8	32*	32	32
Gentamicin	32	16	> 128	> 128	> 128	> 128
Kanamycin	> 512	> 512	256	512	512	512
Streptomycin	> 64	> 64	> 64	> 64	> 64	> 64
Carbapenems (beta-lactam)						

[†] Minimum inhibitory concentration (MIC) determined by the broth microdilution method. MICs were confirmed by two or three independent replicates. MIC differences greater than 2-fold with respect to the corresponding wild type strain were considered significant (indicated with an asterisk).

Antibiotic	MIC [†] in µg/ml					
	LESB58		C17		PAER-10821	
	WT	$\Delta ttg2$	WT	$\Delta ttg2$	WT	$\Delta ttg2$
Imipenem	2	2	32	32	32	64
Meropenem	2	2	32	16	16	16
Cephalosporins (beta-lactam)						
Ceftazidime	256	256	64	128	16	32
Penicillins (beta-lactam)						
Piperacillin	256	256	256	> 256	128	256
Piperacillin-tazobactam	128	128	256	> 256	64	64
Ticarcillin	> 256	> 256	> 256	256	64	128
Ticarcillin-clavulanic acid	> 32	> 32	> 32	> 32	> 32	> 32
[†] Minimum inhibitory concentration (MIC) determined by the broth microdilution method. MICs were confirmed by two or three independent replicates. MIC differences greater than 2-fold with respect to the corresponding wild type strain were considered significant (indicated with an asterisk).						

The three *ttg2* mutants were significantly more sensitive (between 4- and 64-fold) than the corresponding wild-type bacteria to colistin, fluoroquinolones, and tetracycline analogues, but not to the other antibiotic classes (Table 1). The mutant susceptibility phenotypes could be reverted by providing an intact copy of the entire PAO1 *ttg2* operon (PA4456-PA4452) in a replicative plasmid, except for colistin. The lack of complementation of the colistin susceptibility phenotype could be due to the effect of the antibiotic erythromycin (used as a selection marker for complemented strains) on the expression of global regulators that may influence colistin susceptibility^{50, 51} or to the overexpression of the *ttg2* operon components (two- to eight-fold with respect to wild type, see Fig. S13) that may also affect the distribution of phospholipids in the OM. Surprisingly, the susceptibility to amikacin significantly decreased for the C17 mutant and an opposite effect was observed for the LESB58 mutant and tobramycin, suggesting a genetic-background component in the effect of the *ttg2* mutation on the susceptibility to these antibiotics.

Discussion

We have performed a structural and functional study of the soluble periplasmic SBP component of the Ttg2 ABC transport system in *P. aeruginosa* (Ttg2D_{Pa_e}) that reveals new facets of this protein family and provides additional insight into the role of this pathway in *P. aeruginosa*. We have first characterized this protein at the molecular level, supporting its predicted role as a phospholipid transporter. The crystal

structure of recombinant Ttg2D_{P_{ae}} (Fig. 1), and MS analysis of protein-ligand complexes formed *in vivo* and *in vitro* (Fig. 3–5), show that this SBP transports four acyl chains. Furthermore, our results demonstrate that the Ttg2 system in *P. aeruginosa* is a general glycerophospholipid transporter with the ability to carry either two phospholipids or a tetra-acyl cardiolipin-like species. Reevaluation of the Ttg2D structure from *P. putida* (PDB 4FCZ) by Ekiert *et al.*²⁷ (PDB 5UWB) had previously suggested but not confirmed the presence of a tetra-acyl, cardiolipin-like lipid in its bulky hydrophobic pocket. This is in contrast with the Ttg2D/MlaC orthologs from *E. coli* (PDB 5WA) and *R. solanacearum* (PDB 2QGU), which seem to bind a single diacylglyceride based on electron density and cavity size. Although, more recently, Hughes *et al.* showed evidences of cardiolipin binding to *E. coli* MlaC³², these have yet to be confirmed, since the methodology used does not allow the distinction between specific binding and co-purification (unspecific). With the data at hand, it thus appears that, among Gram-negative bacteria, the ability of MlaC-family proteins to transport two phospholipids or cardiolipin is exclusive to some taxonomic groups. Phylogenetic and sequence analysis (Fig. 2) suggest that there are other genera in addition to *Pseudomonas* where the Mla system transports two molecules simultaneously, although this needs to be confirmed by further studies. This finding raises the question whether the evolution of this system in these species has been driven by transport efficiency (double cargo) or transport diversity (tetra-acyl in addition to diacyl phospholipids). Furthermore, are the two phospholipids translocated simultaneously by the permease Ttg2B, as it would need to be for a tetra-acyl phospholipid such as cardiolipin? The determination of the structure of additional transport components in other species will be necessary to corroborate our proposed classification and answer these questions.

MS analyses showed that Ttg2D_{P_{ae}} is a highly promiscuous SBP. This protein is indeed able to bind *in vivo* phospholipids with different head groups, particularly PG and PE, and of different chain lengths and degree of unsaturation. Therefore, this system would not only control the global phospholipid content of the OM, but could be also controlling a precise membrane-lipid distribution. Bacterial cells tightly regulate the phospholipid composition of the OM to fortify the permeability barrier against small toxic molecules, including antibiotics. For example, anionic phospholipids like PG interact with membrane proteins and cationic antimicrobials in ways that zwitterionic phospholipids like PE do not; their balance requiring a fine control^{19, 52}. Indeed, the membrane's PE content is a major factor determining the bacterial susceptibility to certain antimicrobial agents^{52, 53}. On the other hand, anionic non-bilayer-forming phospholipids like cardiolipin can strengthen the OM of Gram-negative bacteria against certain antibiotics¹⁹. In Gram-negative bacteria, cardiolipin is mostly located within the IM (the site of its synthesis), but it is also present in the OM where it facilitates proper localization of proteins on the bacterial surface. Known mechanisms of anterograde transport ensure the presence of cardiolipin in the OM⁵⁴, but a retrograde mechanism preventing accumulation in the OM has not been described yet. An increase in the cardiolipin content of the OM could cause an increase in susceptibility to cationic antimicrobial agents⁵⁵. Positively charged antimicrobial peptides and polymyxins have been proposed to promote the clustering of anionic phospholipids leading to phase-boundary defects that transiently breach the permeability barrier of the cell membrane⁵². In *P. aeruginosa*, an organism showing significant

intrinsic resistance to certain antibiotics, the membrane PE:PG:cardiolipin composition is approximately 60%:20%:11%¹⁸. Simultaneous transport of four acyl chains across cell membranes could help control membrane charge balance more efficiently, not only by number but also by inclusion of additional (cardiolipin) species.

Early studies²⁰ (and more recent ones^{56, 57}) of the Ttg2/Mla system supported its role in the maintenance of lipid asymmetry in the Gram-negative OM, by retrograde trafficking of phospholipids from the OM to the cytoplasm through the IM. Conversely, other studies have recently shown that the *E. coli* protein MlaD spontaneously transfers phospholipids to MlaC *in vitro*^{32, 56}, suggesting a transport in the opposite direction (IM-to-OM). In addition, an Mla mutant in *A. baumannii* showed decreased abundance of OM phospholipids and accumulation of newly synthesized phospholipids in the IM³⁰. Unlike most of the ABC exporters, ABC importers in Gram-negative bacteria require periplasmic SBPs that provide specificity and high-affinity. In addition, it is widely accepted that the direction of substrate transport of ABC transporters can be predicted on the basis of both the sequence of the nucleotide-binding component (ATPase)^{57, 58} and the transmembrane-domain fold of the permease component⁵⁹. The close orthologs in *E. coli* and *Mycobacterium tuberculosis* of the *P. aeruginosa* ATPase Ttg2A, MlaF (60% identity) and the Mce protein Mkl (40% identity), respectively, have sequence signatures typically found in prokaryotic ABC import cassettes^{20, 58}. Moreover, the remote homolog TGD3 from *Arabidopsis thaliana* is also a component of an ABC transport system (TGD) that imports phosphatidic acid to the chloroplasts through its outer and inner envelopes⁶⁰. On the other hand, and although operon *ttg2* resembles the classic organization of an ABC importer, there are evidences at the amino acid sequence level supporting evolution toward anterograde function. For instance, structural similarity searches with Dali (see Text S1) for the Ttg2B ortholog protein in *A. baumannii* (MlaE, PDB 6IC4 chains G and H)³⁰, identified as best match a structure of the human ABCA1 (PDB 5XJY), a known ATP-binding cassette cholesterol/phospholipid exporter⁶¹. All things considered, we should not rule out the possibility of anterograde, in addition to retrograde, phospholipid trafficking by the Ttg2/Mla system. One possibility would be that of a countercurrent model⁶², in which different types of phospholipids would exchange between the two membranes obeying to a gradient (Fig. 7). A countercurrent model would explain how asymmetries in membrane lipid distribution might be achieved by the Ttg2/Mla system, but the direction in which each phospholipid is transported would need to be determined. In the anterograde transport proposed for *E. coli*³², MlaC is loaded by MlaD, suggesting that, for this to be efficient, MlaC should not bind free phospholipids. Taken back to the *P. aeruginosa* system, we have shown that Ttg2D_{Pae} can load free cardiolipin *in vitro*, but have no data for the spontaneous binding of two diacyl phospholipids. In this system, the idea that the loading of two molecules may require a helper protein (the MlaD ortholog Ttg2C) seems plausible. This would facilitate a putative mechanism in which the Ttg2 system would contribute to the retrograde transport of single lipids (cardiolipin, maybe diacyl lipids also) as well as to the export, enabled by Ttg2C, of pairs of diacyl lipids to the OM (Fig. 7). The exact function of VacJ (MlaA equivalent) and its relation with the transport in either direction is still to be determined. While *P. aeruginosa*'s *vacJ* gene is located outside the *ttg2* operon, we have data demonstrating that strains lacking this gene share the same phenotype shown

by *ttg2* mutants. In *E. coli*, MlaA forms an active complex with the outer membrane proteins OmpC and OmpF^{22, 24, 63}. However, in *P. aeruginosa* there are no clear orthologs to either of these porins, increasing the singular characteristics of this system in this species and suggesting potential mechanistic differences with the more studied *E. coli* transporter (Fig. 7).

We have provided additional evidence, based on NPN-uptake and antimicrobial susceptibility assays, that the Ttg2 system controls the permeability of the OM in *P. aeruginosa* regardless of genetic background. Cellular studies showed that deletion of Ttg2D specifically increases the susceptibility to polymyxin, fluoroquinolone, chloramphenicol and tetracycline antibiotics in the PAO1 reference strain and in three MDR clinical strains (Fig. 6 and Table 1). This mutant phenotype was observed both in the presence and absence of specific resistance mechanisms providing high-level resistance. For example, PAO1 is a relatively sensitive strain and LESB58 is a MDR strain, and both show diversity in their resistomes⁶⁴. Thus, for the strain and antibiotic panel considered, the increase in susceptibility upon *ttg2* deletion seems to correlate with the antibiotic class rather than with the genetic background. This is in line with the physico-chemical properties of these antimicrobial compounds. Albeit positively charged, colistin is a significantly hydrophobic antibiotic that appears to gain access to the IM by permeating through the OM bilayer, while tetracyclines, chloramphenicol and quinolones use a lipid-mediated or a porin-mediated pathway depending on protonation state⁶⁵. These antibiotic classes are classified within the same group of molecules according to their interactions with the cell permeability barriers⁹. The fact that other relatively hydrophobic antibiotics such as aminoglycosides are unaffected by the disruption of the Ttg2 system speaks in favor of the observed correlation between membrane phospholipid content and specific susceptibility to certain antibiotics^{52, 53}. Another hypothesis that would explain the different impact of Ttg2 disruption on different antibiotic classes would be the possibility that phospholipids carried by the Ttg2 system, particularly cardiolipin, may interact with or stabilize certain efflux pumps in *P. aeruginosa*. A *P. putida* cardiolipin synthesis mutant was more sensitive to several antibiotics and to toluene, probably due to structural alterations in the RND efflux pumps⁶⁶. Indeed, the protein composition of the OM can also have a strong impact on the sensitivity of bacteria to the different antibiotic classes⁶⁵. It was probably the secondary effects of the removal of Ttg2D function that led the authors of another study, based on results on a Ttg2A mutant, to conclude that the function of the Ttg2 system in *P. aeruginosa* was associated with the export of antibiotics such as tetracycline out of the cell³³. Although further investigations are still required, the activity of the Ttg2 system on membrane phospholipid homeostasis appears to be partly responsible for the lower basal susceptibility of *P. aeruginosa* to antimicrobial agents, particularly to polymyxins (see supplementary discussion).

Methods

Bacterial strains

All bacterial strains used in this study are provided in supplementary Table S6 and growth conditions in supplementary Text S1.

Expression, purification and preparation of Ttg2D_{Pae} protein variants

Recombinant Ttg2D from *P. aeruginosa* (Ttg2D_{Pae}) was obtained in the cytoplasm of *Escherichia coli* BL21(DE3) using the pET-based expression system and was purified to >99% purity. A *P. aeruginosa* PAO1 mutant lacking *ttg2D* was used for the homologous expression of a His-tagged variant of the protein in its natural environment and its subsequent purification. Periplasmic protein preparation from *P. aeruginosa* was done by sucrose-lysozyme method⁶⁷. Both protein variants are tagged with a C-terminal 6-histidine tail for purification by affinity chromatography. Detailed methods for proteins expression and production are available in supplementary Text S1. Samples containing both homologously and heterologously produced proteins were desalted with the appropriate buffer on centricon micro concentrators for MS analyses.

Ttg2D_{Pae} structure resolution

Recombinant Ttg2D_{Pae} obtained in *E. coli* was used to produce crystals for structure determination. Methods for crystallization, data collection and structure refinement are available in supplementary Text S1. The data collection, processing, and refinement statistics are given in supplementary Table S1. Atomic coordinates and structure factors have been deposited in the PDB with entry code 6HSY.

Delipidation of purified recombinant Ttg2D_{Pae}

Recombinant protein produced in *E. coli*, diluted 1:1 with 1% TFA, was delipidated using an HPLC system and a C18 column (Phenomenex Jupiter 5U C18 300A) in 0.1% TFA. Protein was eluted with a gradient of acetonitrile, 0.1% TFA (monitored at 214 and 280 nm) and its delipidation was confirmed by native MS analyses (Fig. 4 inset). Delipidated protein was lyophilized, and typically resuspended in 100 mM NaCl, 10 mM Tris-HCl (pH 8.5), to counteract the acidity of TFA, before exchanging the buffer to the desired one.

Native mass spectrometry analysis and identification of bound phospholipids

Native MS experiments were performed using a Synapt G1-HDMS mass spectrometer (Waters, Manchester, UK) at the Mass Spectrometry Core Facility of IRB Barcelona. Prior to the analysis, samples were desalted with 100 mM ammonium acetate on centricon micro concentrator. Samples were infused by automated chip-based nanoelectrospray using a Triversa Nanomate system (Advion BioSciences, Ithaca, NY, USA) as interface. After ion isolation, fragmentation was performed by CID (collision induced dissociation) in the transfer or trap region by applying increasing collision energies. See supplementary material (Text S1) for method details. Three technical replicates were performed for native MS experiments.

Analysis of protein Ttg2D_{Pae} variants and their bound phospholipids was also done under denaturing conditions by electrospray ionization (ESI) MS in both the positive and negative ion mode (see Text S1). Fragmentation analysis of the most abundant and representative glycerophospholipids released from Ttg2D_{Pae} were done under denaturing MS conditions (non-native) in positive mode. Annotation of most

abundant phospholipids present in each sample was done using Lipidomics Gateway (<http://www.lipidmaps.org>) based on the m/z values of MS spectra and according to Oursel et al.⁴⁴ and Gidden et al.⁴⁵ for *E. coli* phospholipids, and Groenewold et al.⁴⁷ for *P. aeruginosa* phospholipids. In addition, to determine the phospholipid composition from both *E. coli* BL21(DE3) and *P. aeruginosa* PAO1, and to identify the most detected phospholipid species by MS, lipidome analysis of intact cells was done according to Angelini et al.⁶⁸ (see Text S1).

Cardiolipin binding to Ttg2D_{Paε} by native mass spectrometry

1.5 mg of cardiolipin CL(18:0)₄ (sodium salt) (Avanti Polar Lipids, Merk, 710334P-25MG) were reconstituted in MeOH, vortexed and sonicated to obtain a homogeneous solution (1.5 mg/ml, 980 μM). Delipidated protein was mixed with cardiolipin at 1:9 molar ratio (26.25 μM:245 μM) and incubated at 37°C for 1 h at 500 rpm. The excess of cardiolipin were cleaned changing the buffer to 200 mM ammonium acetate using centrifugal filter units of 3K cut-off (Amicon Ultra, Millipore). Buffer was exchanged 3 times at 15°C at 13000g. The reaction was directly injected for MS analysis. Three technical replicates were measured. To check the behavior of cardiolipin and its fragmentation patterns in the different MS conditions applied, commercial CL(18:0)₄ has been used as a standard in all assays.

Generation of markerless *ttg2* mutants in MDR *P. aeruginosa* strains and complementations

Markerless *P. aeruginosa* mutants were constructed using a modification of the pGPI-Scel/pDAI-Scel system (Fig. S13) originally developed for bacteria of the genus *Burkholderia* and other MDR Gram-negative organisms^{49, 69}. The bacterial strains and plasmids of the pGPI-Scel/pDAI-Scel system were kindly donated by Uwe Mamat (Leibniz-Center for Medicine and Biosciences, Research Center Borstel, Borstel, Germany) with permission of Miguel A. Valvano (Center for Infection and Immunity, Queen's University, Belfast, UK). The pGPI-Scel-XCm plasmid⁴⁸ was first modified to facilitate the generation of *ttg2* mutants in MDR *P. aeruginosa* strains. Plasmid modifications include replacement of the chloramphenicol resistance cassette by an erythromycin resistance cassette and deletion of a DNA region containing the Pc promoter found in *P. aeruginosa* class 1 integrons (Text S1 and supplementary Table S6 for details). The sequence of the new suicide plasmid vector, pGPI-Scel-XErm, is available through GenBank under the accession number KY368390. For complementation in PAO1, full *ttg2* operon or the codifying region of the *ttg2D* gene were cloned into the broad-host-range cloning vector pBBR1MCS-5 or a variant thereof containing the arabinose promoter, respectively (Table S6). For complementation experiments in MDR strains, the cloning vector pBBR1MCS-5 was first modified to confer resistance to erythromycin (see details in Text S1). Sequence for the new cloning vector, pBBR1MCS-6 is available through GenBank under the accession number KY368389. Complemented strains were obtained by transforming mutant cells with the corresponding pBBR1MCS derivative plasmid. The expression of *ttg2D* in mutant and complemented strains was verified by reverse transcription-PCR (RT-PCR) and quantitative real-time RT-PCR analysis (Text S1 and Fig. S13).

Outer membrane permeabilization assay

Fluorometric assessment of outer membrane permeabilization was done by the 1-*N*-phenylnaphthylamine (NPN) uptake assay as described by Loh et al.⁷⁰ with modifications (Text S1). Since *P. aeruginosa* PAO1 cells have proven to be poorly permeable to NPN⁹, either EDTA (0.2 mM) or colistin (10 µg ml⁻¹) was added to cells to enhance uptake and fluorescence.

Susceptibility to antibiotics and membrane-damaging agents

Antimicrobial susceptibility to a range of antibiotics was tested by determination of the minimum inhibitory concentration (MIC) using the broth microdilution method or Etest (Biomerieux) strips, following the Clinical and Laboratory Standards Institute (CLSI) guidelines^{71,72} and manufacturer's instructions, respectively (see Text S1 for details). MIC differences higher than 2-fold were considered significant changes in antibiotic susceptibility. Low-level, basal resistance to a given antibiotic was defined as that of an organism lacking acquired mechanisms of resistance to that antibiotic and displaying a MIC above the common range for the susceptible population⁷³. Clinical susceptibility breakpoints against *Pseudomonas sp.* for selected antibiotics have been established by EUCAST⁷⁴. Tolerance to organic solvents and SDS/EDTA was assessed using solvent overlaid-solid medium and MIC assays, respectively (Text S1).

Biofilm formation

Biofilm quantification in 96-well microtiter plate by the crystal violet assay was done as previously described⁷⁵ with modifications (supplementary Text S1).

Bioinformatic analysis

Details are provided in the supplementary Text S1.

Declarations

Acknowledgements

This work has been supported by funding under the Seventh Research Framework Programme of the European Union (ref. HEALTH-F3-2009-223101) and the Spanish Ministry of Science, Innovation and Universities (ref. BIO2015-66674-R). The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication. We acknowledge the European Synchrotron Radiation Facility for provision of synchrotron radiation facilities and thank the staff of ID23-1 for assistance in using the beamline. Part of the mass spectrometry experiments were performed at UAB's proteomics facility SePBioEs.

Contributions

DY, MDL, LC, OCS, AM, MFN and MV conducted the experiments; DY, MDL, LC, OCS, MV, IG and XD designed the experiments and participated in the analysis and interpretation of experimental data; DY, LC,

OCS and MDL wrote the paper; MV, IG and XD supervised research and revised the manuscript.

Competing interests

The authors declare no competing interests.

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Supplementary Materials

Text S1 with supplementary methods

Text S2 with supplementary discussion

Tables S1 – S6

Figures S1 – S13

Supplementary references

Figures

Figure 1

Ttg2DPae binds two phospholipids simultaneously. (A) Crystal structure of Ttg2DPae with two PG (16:0/cy17:0) bound. The feature-enhanced electron-density map around the modeled lipids, shown as a mesh, is contoured at 1.5σ . The cartoon representation of the protein is colored according to the CATH domains: domain 1 in blue and domain 2 in orange, dark tones for segments 1 and light tones for segments 2 in each domain (see Fig. S1). Left panel emphasizes the “decanter” shape of the structure,

and right panel the lipid binding. (B) Structures of Ttg2D from *P. aeruginosa* (orange, left panel) and *R. solanacearum* (slate, right panel). The cavities of both proteins are shown as semi-transparent surfaces (side-view from the right of A). (C) Interactions between the lipid head group and the protein. Hydrogen bonds are shown as black dotted lines. (D) Protein motions along the normal mode 7. The colors represent the B-factors (spectrum blue to red for lowest to highest values).

Figure 2

Sequence and structural diversity among MlaC family proteins. (A) Superposition of the structure of Ttg2D from *P. aeruginosa* with known structures of ortholog proteins (PDB codes indicated). Left panel shows superposition of whole structures and right panel highlights the superposition of helix $\alpha 8$. (B) Phylogeny of 151 representative amino-acid sequences belonging to the Pfam family MlaC (PF05494) and identified across different Gram-negative species (bacterial classes are shown with different colors). The maximum likelihood tree was calculated with the LG+G+F model on MEGA 7, and visualized and annotated with iTOL. UniProt codes are used to identify each sequence, and those proteins with known 3D structure are indicated in red (P0ADV7 in *E. coli*, Q8XV73 in *R. solanacearum*, Q88P91 in *P. putida* and Q9HVV4 in *P. aeruginosa*). The shaded branch corresponds to proteins that we predict to bind two diacyl lipids. This branch comprises sequences from different species belonging to four orders of Gamma-proteobacteria (Pseudomonadales, Alteromonadales, Cellvibrionales and Oceanospirillales). (C) The aligned sequence logo shows a signature motif that distinguishes the sub-set of sequences corresponding to the shaded branch of the tree (18 sequences) from the whole set of 151 representative sequences of the MlaC family. At a given position, the height of a residue is proportional to its frequency. Residue numbering according to the sequence in *P. aeruginosa*; positions 189 and 195 are highlighted.

Figure 3

Ttg2DPae is a promiscuous phospholipid-binding protein. (A) Mass spectrum of Ttg2DPae (produced in *E. coli*) under native conditions (native MS), nano-electrospray ionization (nESI) in positive ion mode. The two major charge states (z=9 and z=10) correspond to a deconvoluted average mass that matches the MW of the recombinant protein (P) (22836.78 Da) plus two phospholipid (2PL) molecules. The peaks m/z 500-1500 correspond to partially denatured proteins and the peaks m/z 3000-6000 correlate with protein aggregates formed in gas phase. (B) Fragmentation mass spectrum for the isolated ion at m/z = 2700 (z = 9); PLs are expected in the range 600-800 Da. (C) Zoom of the MS/MS spectrum in positive ion mode showing the most abundant glycerophospholipids released from Ttg2DPae under non-denaturing MS dissociation conditions. The major PLs detected show a loss of 141 Da, after the MS/MS experiment,

that corresponds to the release of a phosphoethanolamine (PE) moiety. (D) Negative ion mode mass spectrum under denaturing conditions of glycerophospholipids released by Ttg2DPae (FT resolution 100 k and accuracy <3ppm). With this ionization mode, phosphatidylglycerol (PG) species are mostly detected. A detailed list of detected peaks is shown in Table S3. We propose that peaks m/z 1050-1160 correspond to *E. coli* cardiolipin species which have lost one of the fatty acids at sn-1 or sn-2. (E) Structure of the most common species of PE and cardiolipin in *E. coli* showing the most favorable fragmentation sites. PGP: phosphatidylglycerol-phosphate.

Figure 4

Ttg2DPae binds cardiolipin in vitro. Mass spectrum under native conditions in positive ion mode of delipidated protein Ttg2DPae (P) produced in *E. coli* (MW 22836.78 Da) after incubation with free cardiolipin CL(18:0)4 (MW 1466.06 Da) at a ratio 1:9 (P:CL). The inset shows the ion chromatogram of the delipidated Ttg2DPae sample before incubation.

Figure 5

Phospholipids carried by Ttg2DPae in the periplasm of *P. aeruginosa*. (A) Schematic representation of the production of homologous protein Ttg2DPae with a 6xHis in the periplasm of a *P. aeruginosa* PAO1 mutant strain lacking the wild type Ttg2D component of the Ttg2 system. A pBBR1MCS derivative plasmid was used for the expression of the *ttg2D* gene under the control of the arabinose promoter from pBAD. (B) Positive ion mode, non-denaturing mass spectrum of protein Ttg2DPae produced in *P. aeruginosa*. The His-tagged protein has a molecular weight of 22140.37 Da after cleavage of the

predicted signal peptide. Purified protein is shown in the inset. (C) Fragment ion mass spectra of isolated ions around $m/z = 2626$ ($z = 9$) using varying transfer collision energies (CE). These results confirm that a fraction of the Ttg2DPae (P) population purified from the periplasmic space of *P. aeruginosa* is complexed with two phospholipids (PL) and another fraction binds cardiolipins (CL). The most abundant cardiolipin species in strain PAO1 based on a lipidomic analysis (Fig. S10B) are CL(68:2) with MW 1405.0 Da and CL(70:5) with MW 1427.0.

Figure 6

Phenotypic changes in the Δ ttg2D *P. aeruginosa* mutant denote destabilization of its outer membrane. (A and B) Ability of EDTA and colistin to permeabilize the outer membrane (NPN assay) of the native, mutant and complemented PAO1. Data presented are the mean $\pm$ s.d. from 3 independent experiments. (C) Relative change of the Δ ttg2D mutant MIC (minimum inhibitory concentration) for antibiotics of different classes grouped according to their cell entry mechanism. Fold changes were determined with respect to the PAO1 wild type, represented as dotted lines (raw data in Table S3). (D) Growth in LBMg plates overlaid with 100% p-xylene. Under this condition the growth was assessed following incubation at 37°C for 24h. The image is representative of duplicate experiments. (E) Relative biofilm formation

determined by crystal violet (CV) staining for Δ tgt2D mutant and control strains in LB medium with and without EDTA. Data presented are the mean $\pm$ s.d. ($n = 8$). Asterisks denote the significance of the data between groups (one-way ANOVA with Tukey's multiple comparison test). In all panels pBBR1 indicates insertion of pBBR1MCS-5 vector alone as a control. Source data are provided as a Source Data file.

Figure 7

Proposed model of the Ttg2 system in *P. aeruginosa*. The soluble substrate binding protein Ttg2DPae, together with the inner membrane complex Ttg2ABCE, transports mislocalised glycerophospholipids between the two cell membranes through the periplasm of *P. aeruginosa*. It is not yet known if the VacJ component of this system, which is thought to deliver the phospholipids to Ttg2DPae, forms a complex with specific porins, as in *E. coli*, to extract the lipids from the outer membrane. Ttg2DPae can carry two glycerophospholipids (e.g phosphoethanolamine PE or phosphatidylglycerol PG) and, based on structural and mass spectrometry data, it could accommodate a tetra-acyl phospholipid such as cardiolipin (CL). Considering recent studies^{30, 32}, the structural signatures of the ATPase and permease models from the ortholog Mla system in *E. coli*²⁷, characteristic of importer and exporter ABC cassettes, we propose, in addition to a role in retrograde phospholipid trafficking, a second potential mode of action as an

anterograde trafficking system (dashed lines) that would contribute to the maintenance of phospholipid distribution asymmetry. The direction in which this system transports the different phospholipid classes remains to be explored.

Supplementary Files

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